EVALUATION OF MANNOSE BINDING LECTIN SERUM LEVEL OF DIABETIC PATIENTS

By

Ola Bashir Sallam

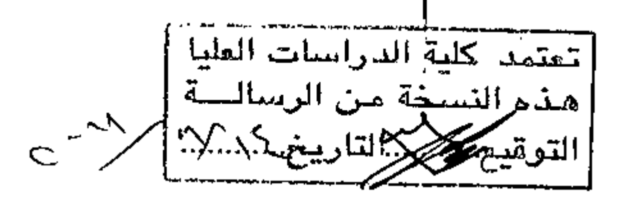
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This Thesis was Submitted in Partial Fulfillment of the Requirements for the Master's Degree of Science in Clinical Pharmacy

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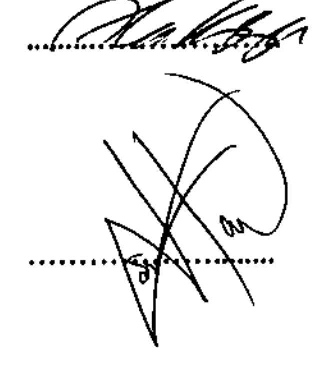
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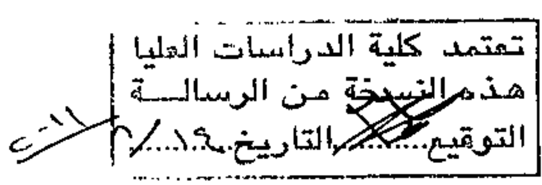
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COMMITTEE DECISION

This Thesis/Dissertation (Evaluation of Mannose Binding Lectin Serum Level of Diabetic patients) was Successfully Defended and Approved on 8 August 2011

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DEDICATION

To my mother and father who lighted my life

To my husband who always supported me

To my beloved sister Haneen who stood beside me and supported me

To all my family

To my teachers Dr. Yasser and Dr. Mohammad who were more than teachers

To all those who supported me and helped me

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LIST OF ABBREVIATIONS

ACEIs	Angiotensin converting enzyme inhibitor
ADA	American Diabetes Association
AGEs	advanced glycosylation end products
ARBs	Angiotensin receptor blockers
Asp	Aspartate
BMI	Body mass index
CAD	Coronary artery disease
CCBs	Calcium channel blockers
CrCl	Creatinine clearance
CRD	Carbohydrate-recognition domain
CVD	Cardiovascular disease
Cys	Cystiene
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DR	diabetic retinopathy
ELISA	Enzyme linked immuno sorbent assay
ESRD	end-stage renal disease
FGF	fibroblast growth factor
GFR	Glomerular filtration rate
GlcNAc	N-acetyl-d-glucosamine
Glu	Glutamate
Gly	Glycine
HbA1c	Glycosylated hemoglobin
HDL	high-density lipoprote
HRP	horseradish peroxidase
IL	interleukin
MASPs	mannose-associated serine proteases
MBL	Mannose binding lectin
MO	macular oedema
OD	optical density
OR	Odds ratio
PDGF	platelet-derived growth factor
PDR	proliferative diabetic retinopathy
rpm	round per minute
TMB	tetramethylbenzidine
UAE	urinary albumin excretion
UKPDS	UK Prospective Diabetes Study
VEGF	vascular endothelial growth factor

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EVALUATION OF MANNOSE BINDING LECTIN SERUM LEVEL OF DIABETIC PATIENTS

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ABSTRACT

Mannose binding lectin (MBL) plays a major role in the innate immunity. Some studies found that it has a role in insulin resistance and the risk of developing diabetes mellitus. Other studies found a relationship between MBL and diabetes complications.
The aim of this study was to evaluate the level of MBL in serum of Jordanian diabetic patients compared to non-diabetics and to explore possible relationship between MBL and diabetes complications.
Sixty five diabetic patients and fifteen non-diabetics were enrolled in the study. Blood samples were collected and MBL serum level was measured using ELISA technique. Serum creainine was also measured to assess kidney function.
Higher serum MBL levels were found in diabetic patients compared to non-diabetics, but the difference was not statistically significant.
Higher serum MBL levels were associated with diabetic nephropathy represented by albuninuria level and different types of diabetic retinopathy, these associations were statistically significant.
In conclusion, high MBL serum levels may play an important role in diabetic patients and may help to predict the risk of developing diabetes complications which allow us to take strict prophylactic and treartment measurements in such populations.

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1. INTRODUCTION

1.1 The mannose binding lectin (MBL) protein

1.1.1 Structure of MBL

MBL is an acute phase protein primarily produced by the liver. The basic structural unit of MBL is a homotrimer of MBL peptides (sub-units) that self-associate into a collagen-like triple helix (Fig. 1). Each peptide possesses a C-terminal calcium-dependent lectin domain, which recognizes repetitive oligosaccharide moieties present on a wide array of pathogens (Kilpatrick *et al.*, 2002; Holmskov *et al.*, 2003). Functional MBL circulates as higher order multimers (tetramers, pentamers and hexamers) of the structural (homotrimeric) unit. The higher order structure allows high-affinity interaction between MBL lectin domains and microbial oligosaccharides, resulting in conformational change of the MBL multimer and activation of associated molecules, the mannose-associated serine proteases (MASP) (Worthley *et al.*, 2005).

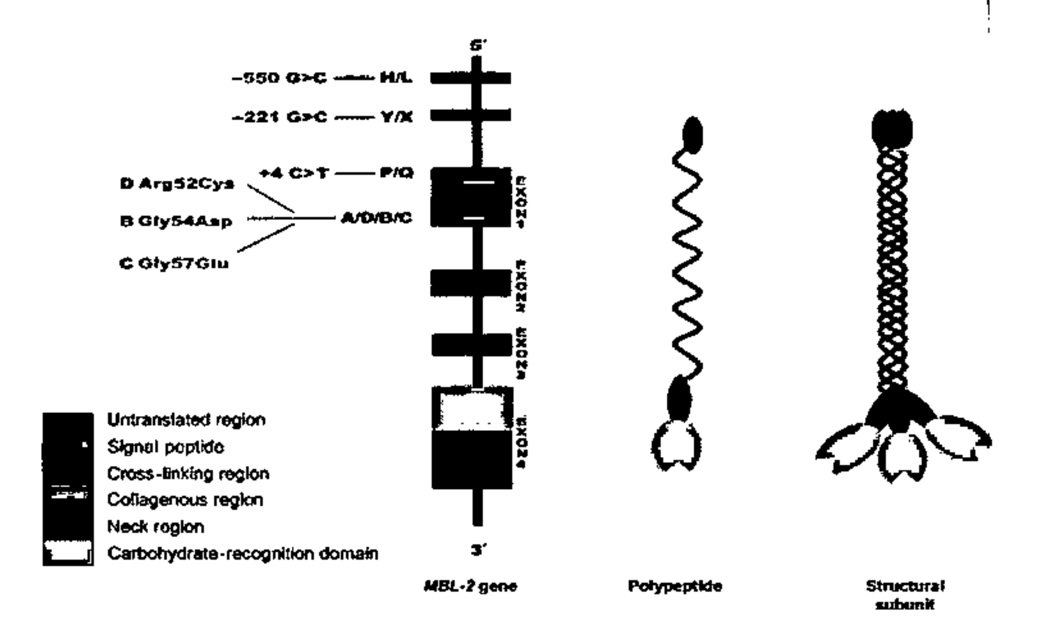


Fig. (1): Structure of human MBL-2 gene and the encoded protein product. (Dommet *et al.*, 2006)

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1.1.2 Site of MBL production

MBL was first isolated and cloned from hepatocytes, and the liver is considered to be the principal site of MBL synthesis (Summerfield JA, Taylor ME, 1986). However, several clinical and experimental studies suggest that MBL may be synthesized in significant amounts by non-hepatic tissues. If MBL were exclusively synthesized by the liver then one would expect MBL levels to fall with advancing hepatic failure. However, existing data suggest that the situation *in vivo* is more complicated. This was evaluated in two studies on patients with advanced liver disease (Ryley *et al.*, 1993; Homann *et al.* 1995). The first found that in chronic liver disease, stainable liver MBL did not correlate with serum MBL levels (Ryley *et al.*, 1993). The other study showed that serum MBL was significantly elevated in patients with cirrhosis (Homann *et al.* 1995). These observations suggest either that there is significant extra-hepatic contribution to serum MBL, or that there is substantial hepatic reserve and thus MBL sufficiency is maintained even during relative hepatic decompensation.

Experimental data support the concept of non-hepatic MBL synthesis. Human monocytes and monocyte-derived dendritic cells are both capable of synthesizing MBL *in vitro* (Downing *et al.*, 2003). Several rodent studies have also demonstrated extra-hepatic expression of MBL, with the sites responsible including small intestine, kidney, as well as in lymphoid and myeloid cells (Morio *et al.*, 1997; Uemura *et al.*, 2002; Wagner *et al.*, 2003). The relative clinical importance of hepatic and non-hepatic MBL synthesis remains unclear.

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1.1.3 Functions of MBL

MBL binds carbohydrates in the presence of Ca^{2+} through the C-terminal carbohydrate-recognition domain (CRD) (Drickamer *et al.*, 1992; Weis *et al.*, 1992; Sheriff *et al.* 1994). The CRD is able to form bonds with hydroxyl groups on specific ligands, including mannose, N-acetyl-d-glucosamine (GlcNAc), N-acetyl-mannosamine, fucose, and glucose. These carbohydrates are found on pathologic microorganisms, including bacteria, fungi, parasitic protozoans, and viruses. CRD also recognizes molecular structures of dying host cells, including nucleic acids and the metalloproteases, meprin alpha and beta (Garred *et al.*, 2008). Carbohydrates that are found on mammalian glycoproteins, such as D-galactose and sialic acid, have no affinity for MBL. Thus, MBL is able to bind to microbes or unwanted material through binding to ligands expressed on their surfaces and it triggers the activation of the lectin pathway, but it avoids recognition of human glycoproteins because of the low affinity to such structures.

It is now recognized that MBL have a role in processes as diverse as complement activation, promotion of complement-independent opsonophagocytosis, modulation of inflammation, recognition of altered self-structures and apoptotic cell clearance. A brief description of these functions is discussed here.

A. MBL and complement activation

The complement system can be activated through three pathways: the classical pathway, the alternative pathway, and the lectin pathway (figure 2). It is now clear that MBL activates a novel pathway of complement, often termed the MBL or the lectin pathway, in an antibody- and C1-independent fashion as illustrated in Figure 2. This functional

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activity reflects the fact that MBL circulates in association with a group of MBL-associated serine proteases (the so-called MASPs) (Matsushita M, Fujita T, 1992). Current understanding suggests that on binding to microorganisms, autoactivation of MASP-2 occurs, permitting cleavage of C4 and C2 to form a C3 convertase, which is indistinguishable in specificity from the convertases found in the other two activation pathways of complement (Feinberg *et al.*, 2003).

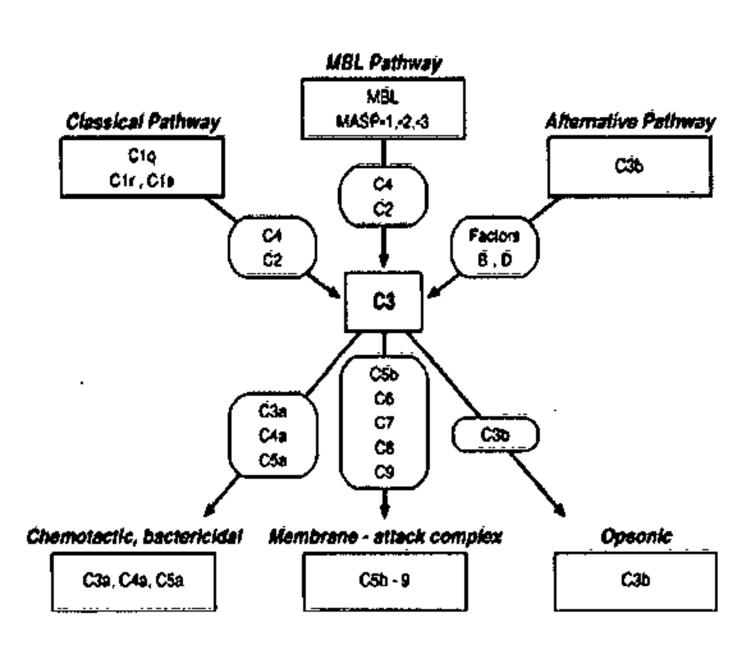


Fig (2): Complement activation pathways.

B. MBL and Opsonophagocytosis

In 1981, studies linked an opsonic deficiency found in children, which was associated with recurrent infections and diarrhea, to the complement system by demonstrating that sera with the deficiency deposited less C3b on yeast surfaces (Turner *et al.*, 1981). However, it was not until 1989 that the common opsonic defect was found to be

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associated with low levels of the mannose-binding protein, which is now referred to as MBL (Super *et al.*, 1989).

C. MBL in inflammation

The role of MBL as a modulator of inflammation appears to be complex and, accordingly, its mechanism of action remains unexplained. One possible explanation is that MBL is able to trigger proinflammatory cytokine release from monocytes (Soell *et al.*, 1995; Chaka *et al.*, 1997). This concept was addressed in a study by Jack *et al* (2001) using *Neisseria meningitidis* incubated with increasing concentrations of MBL before being added to MBL-deficient whole blood. Release of tumor necrosis factor α, interleukin (IL)-1b and IL-6 from monocytes was enhanced at MBL concentrations below 4 mg/ml but suppressed at higher concentrations.

D. The role of MBL in the recognition of altered self and apoptosis

A role for MBL in the clearance of apoptotic cells was first proposed by Ogden *et al.* (2001). MBL was found to bind directly to apoptotic cells that expose terminal sugars of cytoskeletal proteins, thereby permitting their recognition and directly facilitating their phagocytosis by macrophages.

Defects in the clearance of apoptotic cells have been implicated in the pathogenesis of certain autoimmune conditions, although the precise role of MBL, if any, remains elusive. For example, in 2005, Stuart *et al.* reported that although MBL-deficient mice displayed defective apoptotic cell clearance, they did not develop autoimmune diseases. In animal studies, MBL has been implicated in the pathophysiology of ischemia reperfusion injury due to its ability to recognize altered self-structures. Busche *et al* (2008) have proposed the lectin pathway as a mediator of this process in certain organs,

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and the absence of MBL/MASP pathway activation appears to afford protection in these disease models (Hart *et al*., 2005; Walsh *et al*., 2005). However, the relevance of these findings to human health needs to be established.

1.1.4 Genetics of human MBL

There are two MBL genes in humans; MBL1 gene, a pseudogene that does not produce a functional protein, and MBL2 gene which encodes the protein product. The functional MBL2 gene is located on the long arm of chromosome 10 and comprises four exons. Gene expression is primarily regulated by several consensus elements in the promoter region (Garred, P. 2008; Naito *et al*., 1999).

The normal allele is called allele A. To date, three point mutations have been identified, all in exon 1 (Sumiya *et al*., 1991; Lipscombe *et al*., 1992; Madsen *et al*., 1994) : Allele B (codon 54, GGC to GAC (Gly→Asp)) , Allele C (codon 57, GGA to GAA (Gly→Glu)) and Allele D (codon 52, CGT to TGT (Arg→Cys))

The effects of these exon 1 mutations on the protein are believed to impair oligomerization and lead to a functional deficiency (Garred *et al*., 2003).

Coding mutations are common; in an Australian study of 236 volunteers, the prevalence of coding wild-type genotype (*A/A*) was 57.6%, coding mutation heterozygosity 34.8% (*A/D* 11%, *A/B* 19.9%, *A/C* 3.8%), and coding mutation homozygosity 7.6% (*B/B* 2.1%, *B/C* 2.1%, *B/D* 2.5%, *D/D* 0.9%) (Minchinton *et al*., 2002)

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Serum MBL levels, however, vary substantially within each coding genotype. Additional polymorphisms in the promoter and 5′-untranslated regions of the *MBL2* gene partly explain this variation (Madsen *et al.*, 1995; Mead *et al.*, 1997; Garred *et al.*, 2003).

1.2 Type 2 diabetes mellitus

Type 2 diabetes mellitus (DM) is one of the world's most common diseases. Diabetes mellitus, is defined by the American Diabetes Association (ADA) as a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association, Diagnosis and Classification of Diabetes Mellitus, 2010). Type 2 diabetes, or adult-onset diabetes, encompasses individuals who have insulin resistance and usually have relative (rather than absolute) insulin deficiency (American Diabetes Association (ADA), Diagnosis and Classification of Diabetes Mellitus, 2010). ADA Criteria for the diagnosis of DM is shown in table 1 (ADA, standards of medical care in diabetes 2010).

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Table 1: Criteria for the diagnosis of diabetes

1.	A1C ≥6.5%. The test should be performed in a laboratory using a method that is NGSP certified and standardized to the DCCT assay.*
	OR
2.	FPG ≥126 mg/dl (7.0 mmol/l). Fasting is defined as no caloric intake for at least 8 h.*
	OR
3.	Two-hour plasma glucose ≥200 mg/dl (11.1 mmol/l) during an OGTT. The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.*
	OR
4.	In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dl (11.1 mmol/l).

*In the absence of unequivocal hyperglycemia, criteria 1–3 should be confirmed by repeat testing.

According to the 2011 National Diabetes Fact Sheet, 25.8 million children and adults in the United States (8.3% of population) have DM.

In Jordan, the prevalence of DM is much higher, the overall prevalence of DM is 17.1%; in addition, impaired glucose tolerance defined as a fasting serum glucose level of ≥6.1 mmol/l (110 mg/dl) but < mmol/l (Report of the Expert Committee, 1997) was found in 7.8% of the study population (Ajlouni *et al* 2008).

Multiple risk factors for the development of type 2 DM have been identified, including family history (i.e., parents or siblings with diabetes); obesity (i.e., ≥20% over ideal body weight, or body mass index (BMI) ≥25 kg/m^2); habitual physical inactivity; race or ethnicity; previously identified impaired glucose tolerance or impaired fasting glucose; hypertension (≥140/90 mm Hg in adults); high-density lipoprotein (HDL) cholesterol ≤35 mg/dL and/or a triglyceride level ≥250 mg/dL; history of gestational DM or delivery of a baby weighing >4 kg (9 lb); history of vascular disease; presence of acanthosis nigricans; and polycystic ovary disease (Dipiro *et al.*, 2008).

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Long-term complications of diabetes include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers and amputations; and autonomic neuropathy causing gastrointestinal, genitourinary and cardiovascular symptoms and sexual dysfunction. Patients with diabetes have an increased incidence of atherosclerotic cardiovascular, peripheral arterial and cerebrovascular disease. Hypertension and abnormalities of lipoprotein metabolism are often found in people with diabetes (American Diabetes Association, Diagnosis and Classification of Diabetes Mellitus, 2010)

1.2.1 DM Pathogenesis

Type 2 diabetes is characterized by impaired insulin secretion and resistance to insulin action. In the presence of insulin resistance, glucose utilization by tissues is impaired, hepatic glucose production is increased, and excess glucose accumulates in the circulation. This hyperglycemia stimulates the pancreas to produce more insulin in an attempt to overcome insulin resistance. The simultaneous elevation of both glucose and insulin levels is strongly suggestive of insulin resistance. Genetic predisposition may play a role in the development of type 2 diabetes (Dipiro *et al.*, 2008).

Basal insulin levels are typically normal or elevated at diagnosis. First- or early-phase insulin release in response to glucose often is reduced and pulsatile insulin secretion is absent, resulting in postprandial hyperglycemia. The effects of other insulinotropic substances such as incretin hormones, which contribute to meal-stimulated insulin release, are also altered.Over time; β-cells lose their ability to respond to elevated glucose concentrations, leading to increasing loss of glucose control. In patients with severe

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hyperglycemia, the amount of insulin secreted in response to glucose is diminished and insulin resistance is worsened (glucose toxicity) (Dipiro *et al.*, 2008).

Most individuals with type 2 exhibit decreased tissue responsiveness to insulin. Overeating and/or hyperglycemia may contribute to hyperinsulinemia, which over time may lead to a decrease in or downregulation of the number of insulin receptors on the surface of target tissues and organs. Evidence suggests that decreased peripheral glucose uptake and utilization in muscle is the primary site of insulin resistance and results in prolonged postprandial hyperglycemia. Resistance may be secondary to decreased numbers of insulin receptors on the cell surface, decreased affinity of receptors for insulin, or defects in insulin signaling and action that follows receptor binding. Defects in insulin signaling and action are referred to as postreceptor or postbinding defects and are likely to be the primary sites of insulin resistance (Dipiro *et al.*, 2008).

1.3 Diabetes complications

Complications of diabetes can be largely divided into macrovascular and microvascular complications. The macrovascular complications include cerebrovascular disease, coronary heart disease, and peripheral vascular disease. The microvascular complications include diabetic retinopathy (DR), diabetic neuropathy, and diabetic nephropathy.

1.3.1 Diabetic nephropathy (DN)

Diabetes has become the most common single cause of end-stage renal disease (ESRD) in the U.S. and Europe; In the U.S., diabetic nephropathy accounts for about 40% of new cases of ESRD. About 20–30% of patients with type 1 or type 2 diabetes develop evidence of nephropathy, but in type 2 diabetes, a considerably smaller fraction of these

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progresses to ESRD. However, because of the much greater prevalence of type 2 diabetes, such patients constitute over half of those diabetic patients currently starting on dialysis (American diabetes association, Diabetic Nephropathy, 2002).

The earliest clinical evidence of diabetic nephropathy is the appearance of low but abnormal levels (≥30 mg/day or 20 μg/min) of albumin in the urine, referred to as microalbuminuria, and patients with microalbuminuria are referred to as having incipient nephropathy. Without specific interventions, around 80% of subjects with type 1 diabetes who develop sustained microalbuminuria have their urinary albumin excretion increase at a rate of approximately 10–20% per year to the stage of overt nephropathy or clinical albuminuria (≥300 mg/24 h or ≥200 μg/min) over a period of 10–15 years, with hypertension also developing along the way. Once overt nephropathy occurs, without specific interventions, the glomerular filtration rate (GFR) gradually falls over a period of several years at a rate that is highly variable from individual to individual (2–20 ml/ min/ year). ESRD develops in 50% of type 1 diabetic individuals with overt nephropathy within 10 years and in ≥75% by 20 years. A higher proportion of individuals with type 2 diabetes are found to have microalbuminuria and overt nephropathy shortly after the diagnosis of their diabetes, because diabetes is actually present for many years before the diagnosis is made and also because the presence of albuminuria may be less specific for the presence of diabetic nephropathy, as shown by biopsy studies. Without specific interventions, 20–40% of type 2 patients with microalbuminuria progress to overt nephropathy, but by 20 years after onset of overt nephropathy, only 20% will have progressed to ESRD (American diabetes association, Diabetic Nephropathy, 2002).

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In addition to its being the earliest manifestation of nephropathy, albuminuria is a marker of greatly increased cardiovascular morbidity and mortality for patients with either type 1 or type 2 diabetes. Thus, the finding of microalbuminuria is an indication for screening for possible vascular disease and aggressive intervention to reduce all cardiovascular risk factors (e.g., lowering of LDL cholesterol, antihypertensive therapy, cessation of smoking, institution of exercise, etc (American diabetes association, Diabetic Nephropathy, 2002).

Some Pathologic abnormalities are noted in patients with long-standing diabetes mellitus before the onset of microalbuminuria. There are three major histologic changes in the glomeruli in diabetic nephropathy: mesangial expansion; glomerular basement membrane thickening; and glomerular sclerosis (Fioretto *et al.*, 1992; Adler S, 2004).

The last abnormality, which may have a nodular appearance (the Kimmelstiel-Wilson lesion), is often associated with hyaline deposits in the glomerular arterioles (reflecting the insinuation of plasma proteins such as fibrin, albumin, immunoglobulins, and complement into the vascular wall) (Fioretto *et al.*, 1992; Tervaert *et al.*, 2010) . These different histologic patterns appear to have similar prognostic significance (Nasr *et al.*, 2007).

Diabetic nephropathy has been didactically categorized into stages based on the values of urinary albumin excretion (UAE): microalbuminuria and macroalbuminuria. The cutoff values adopted by the American Diabetes Association (14) (timed, 24-h, and spot urine collection) for the diagnosis of micro- and macroalbuminuria are depicted in Table 2.

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Table (2): Definitions of abnormalities in albumin excretion

Stage	Urine with marked time (µg/min)*	24-hour urine (mg/24 h)*	Random urine sample	
			Albumin concentration (mg/l)**	Albumin/creatinine ratio (mg/g)*
Normoalbuminuria	< 20	< 30	< 17	< 30
Microalbuminuria	20 – 199	30 – 299	17 a 173	30 – 299
Macroalbuminuria	≥ 200	≥ 300	≥ 174	≥ 300

* Values according to the American Diabetes Association
** Gross et al., Diabetes Care 2005.

Although the measurement of albuminuria is essential to diagnose DN, there are some patients who present decreased glomerular filtration rate (GFR) when UAE values are normal. Based on this, the classification of the National Kidney Foundation can also be used to stage chronic kidney disease in these patients (Table 3) (American Diabetes Association, Standards of medical care in diabetes, 2009) .. It is recommended that GFR

Table (3): Chronic kidney disease stages

Stage	Description	GFR (ml/min/1.73 m²)
1	Renal damage* with GFR N or ↑	≥90
2	Renal damage* with GFR slightly ↓	60-89
3	GFR moderately ↓	30-59
4	GFR severely ↓	15-29
5	End stage chronic renal failure	<15 or dialysis

*Renal damage is defined by abnormalities in the urine and blood tests, imaging exams or in pathology
GFR = glomerular filtration rate

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1.3.1.1 Risk factors and pathogenesis of diabetic nephropathy

Diabetic nephropathy develops in, at most, 40% of patients with diabetes, even when high glucose levels are maintained for long periods of time. This observation raised the concept that a subset of patients has an increased susceptibility to diabetic nephropathy. Furthermore, epidemiological and familial studies have demonstrated that genetic susceptibility contributes to the development of diabetic nephropathy in patients with both type 1 and type 2 diabetes. The main potentially modifiable diabetic nephropathy initiation and progression factors in susceptible individuals are sustained hyperglycemia and hypertension. Other putative risk factors are glomerular hyperfiltration, smoking, dyslipidemia, proteinuria levels, and dietary factors such as the amount and source of protein and fat in the diet (Gross *et al.*, 2005).

Pathology: Diabetes causes unique changes in kidney structure. Classic glomerulosclerosis is characterized by increased glomerular basement membrane width, diffuse mesangial sclerosis, hyalinosis, microaneurysm, and hyaline arteriosclerosis. Tubular and changes are also present (Gross *et al.*, 2005).

1.3.1.1.1 Effect of glycemic control

The diabetes Control and Complications Trial and the United Kingdom Prospective Diabetes Study have shown definitively that intensive diabetes therapy can significantly reduce the ris of the development of microalbuminuria and overt nephropathy in people with diabetes (ADA, diabetic nephropathy, 2002).

1.3.1.1.2 Effect of hypertension control

In patients with type 2 diabetes, hypertension is present at the time of diagnosis of diabetes in about one-third of patients. Both systolic and diastolic hypertension markedly

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accelerate the progression of diabetic nephropathy, and aggressive antihypertensive management is able to greatly decrease the rate of fall of GFR.

Appropriate antihypertensive intervention can significantly increase the median life expectancy in patients with type 1 diabetes, with a reduction in mortality from 94 to 45% and a reduction in the need for dialysis and transplantation from 73 to 31% 16 years after the development of overt nephropathy(ADA, diabetic nephropathy, 2002).

1.3.1.1.3 Effect of use of antihypertensive agents

Many studies have shown that in hypertensive patients with type 1 diabetes, ACE inhibitors and ARBs can reduce the level of albuminuria and can reduce the rate of progression of renal disease to a greater degree than other antihypertensive agents that lower blood pressure by an equal amount. Other studies have shown that there is benefit in reducing the progression of microalbuminuria in normotensive patients with type 1 diabetes and normotensive and hypertensive patients with type 2 diabetes.

Some studies have demonstrated that the non-dihydropyridine calcium channel blocker (NDCCB) classes of calcium-channel blockers can reduce the level of albuminuria, but no studies to date have demonstrated a reduction in the rate of fall of GFR with their use(ADA, diabetic nephropathy, 2002).

1.3.2 Diabetic retinopathy

Diabetes has many manifestations in the eye, of which cataracts and diabetic retinopathy are the most significant cause of visual impairment and blindness, and people with diabetes are 25 times more likely than the general population to become blind (National Society to Prevent Blindness, 1980). In developed countries, diabetic eye disease represents the leading cause of blindness in adults less than 75 years (National Institutes

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of Health, 1995). More than 60% of type II diabetes sufferers will have evidence of DR during this period (Aiello *et al.*, 1995; Klein *et al.*, 1984). During the first two decades of disease, nearly all patients with type 1 diabetes and >60% of patients with type 2 diabetes have retinopathy. Visual impairment as a result of DR has a significant impact on patients' quality of life (brown *et al.*, 1999) and can compromise their ability to manage successfully their disease, which can in turn have a negative impact on the incidence of other diabetic complications and overall life expectancy.

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DR is a progressive disease predominantly affecting the integrity of the microscopic vessels found in the retina. DR can be broadly divided into two clinical stages: nonproliferative and proliferative diabetic retinopathy (PDR). During nonproliferative DR, the earliest visible sign of retinal damage results from abnormal permeability and/or nonperfusion of capillaries, leading to the formation of microaneurysms (Engerman *et al.*, 1989). Abnormal capillary permeability results in the leaking of fluid and solutes into the surrounding retinal tissue, which collects around the macula; this is referred to as macular oedema (MO) and it threatens visual acuity. PDR develops following the occlusion of retinal capillaries leading to retinal ischaemia, which promotes the development of neovascularization, a process by which new blood vessels proliferate on the surface of the retina. However, these vessels are fragile and haemorrhage easily. The resulting accumulation of blood in the vitreous cavity from these haemorrhaging vessels seriously impairs vision. This may be permanent due to further complications such as traction retinal detachment leading to registered blindness. It has been estimated that without treatment for PDR, 50% of all patients will become blind within 5 years following diagnosis (Hamilton *et al.*, 1996).

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1.3.2.1 Risk factors and pathogenesis

The duration of diabetes is probably the strongest predictor for development and progression of retinopathy. Among younger-onset patients with diabetes in the Wisconsin Epidemiologic Study of Diabetic Retinopathy, the prevalence of any retinopathy was 8% at 3 years, 25% at 5 years, 60% at 10 years, and 80% at 15 years (Klein *et al.*, 1984).

Chronic hyperglycemia is thought to be the primary cause of diabetic retinopathy (Frank RN, 2004). Evidence in support for this hypothesis has come from the Diabetes Control and Complications Trial (DCCT), which found that intensive insulin therapy, achieving mean hemoglobin A1C (HbA1C) of 7.9 percent, reduced the incidence of new cases of retinopathy by as much as 76 percent compared with conventional therapy (A1C of 9.9). The United Kingdom Prospective Diabetes Study found similar results in patients with type 2 diabetes; each 1 percent point reduction in A1C was associated with a 37 percent reduction in development of retinopathy. (UK Prospective Diabetes Study (UKPDS) Group, 1998).

While chronic hyperglycemia is the fundamental prerequisite for diabetic retinopathy, much of current research has focused on the hemodynamic, biochemical, and hormonal mechanisms involved in the pathogenesis of this disease process, in an effort to develop new therapeutic targets. Three of the main mechanisms are: impaired autoregulation of retinal blood flow; accumulation of sorbitol within retinal cells; and accumulation of advanced glycosylation end products in the extracellular fluid.

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The data supporting these mechanisms primarily refer to the induction of retinal injury. More advanced retinal disease, including proliferative vascular changes and neovascularization in the setting of retinal ischemia, may be mediated by other mechanisms such as the action of insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) (Frank RN, 2004; Ruberte *et al.*, 2004).

a. Autoregulation of retinal blood flow: Retinal blood flow is constant until the mean arterial pressure is raised about 40 percent above baseline. However, this autoregulatory mechanism is impaired in the presence of hyperglycemia. The ensuing increase in retinal blood flow causes increased shear stress on the retinal blood vessels, which may be a stimulus for the production of vasoactive substances, vascular leakage, and increased fluid accumulation in the outer layers of the retina, resulting in macular edema (www. UpToDate.com).

b. Sorbitol: Glucose that enters cells is metabolized in part to sorbitol via the enzyme aldose reductase; sorbitol is then metabolized to fructose, a process that is relatively slow. Sorbitol accumulation within the cells of the lens is more pronounced with chronic hyperglycemia. It leads to a rise in intracellular osmolality (which causes movement of water into the cells and cell swelling) and to a decrease in intracellular myoinositol, both of which can interfere with cell metabolism. Sorbitol accumulation may also be important in the cataract formation induced by hyperglycemia, because swelling of lens fiber cells can lead to their rupture (www. UpToDate.com).

c. Advanced glycosylation end products: In chronic hyperglycemia, some of the excess glucose combines with free amino acids or serum or tissue proteins. This nonenzymatic

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process initially forms reversible early glycosylation products and later irreversible advanced glycosylation end products (AGEs). Serum AGE concentrations are high in diabetic patients, a change that can lead to tissue accumulation of AGEs, which may cross link with collagen, thereby initiating microvascular complications. AGE accumulation has also been implicated in cataract formation (www. UpToDate.com).

In addition, the interaction between AGEs and their receptor (RAGE) generates reactive oxygen species and subsequently vascular inflammation. (www.UpToDate.com).

Genetic factors: Genetic influences affect the severity of retinopathy. In 372 patients in the DCCT (who had 467 first-degree relatives with diabetes), severe retinopathy was three times more frequent among the relatives of the retinopathy-positive patients than the retinopathy-negative patients (www. UpToDate.com).

1.4 Association between MBL and type 2 diabetes mellitus

Muller *et al* (2010) demonstrated that an allele for *MBL2*, which arises predominately from the promoter SNP rs11003125 (G allele), predicts a higher serum level of MBL2 and is associated with increased risk for type 2 diabetes, increased 2-hour plasma glucose and 2-hour plasma insulin, and decreased insulin secretion in some populations. The study indicates that *MBL2* variants are more likely to influence type 2 diabetes via an effect on insulin secretion rather than on insulin action, suggesting that inflammatory damage in pancreatic β-cell function may be involved. Consistent with these observations, high MBL levels have recently been reported to be associated with high

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A1C levels in the Strong Heart Study, a longitudinal study of cardiovascular disease among Native Americans (Best *et al.*, 2009).

The physiologic mechanisms underlying the association of MBL2 levels with diabetes are unknown; it has previously been shown that MBL2 plays a dual role in modifying inflammatory responses (Fruebis *et al.*, 2001). Deficiency of MBL has been linked to increased risk of developing type 1 diabetes (Araujo *et al.*, 2007), insulin resistance, and obesity (Fernández-Real *et al.*, 2006) as a result of a chronic infectious state or low-grade inflammation. MBL2 could also affect metabolic pathways through stimulating fatty acid oxidation in skeletal muscle (Soell *et al.*, 1995) or reducing release of tumor necrosis factor-α, interleukin-1, and interleukin-6 (Turner *et al.*, 2000). In contrast, increased MBL levels could lead to an overly activated complement system, thereby inducing inflammatory damage or interweaving a complex autoimmune process (Kilpatrick *et al.*, 2000). Consistent with the latter effect, high MBL levels have been associated with increased risk for insulin resistance in pregnancy (Kilpatrick *et al.*, 2000) and late-onset of rheumatoid arthritis (Garred *et al.*, 2000).

Additional studies are needed to investigate the impact of this gene on specific type 2 diabetes related–pathways and disease susceptibility in non–Native American groups.

1.5 MBL and diabetic nephropathy

MBL pathway has come in focus as a potential pathogenic factor in diabetic nephropathy, as normoalbuminuric type 1 diabetics have higher levels of serum MBL than non-diabetic controls, with a stepwise increase in circulating MBL-levels with increasing levels of urinary albumin excretion (UAE) within the normal range (Hansen *et al.*, 2003).

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Data from a recent study showed that a significantly larger proportion of patients with a high MBL genotype had diabetic nephropathy compared with the group with low MBL-genotypes (OR=1.52) (Hansen *et al.*, 2004). Furthermore, in patients with a high MBL genotype the serum MBL levels were significantly higher in nephropathic patients than in patients without nephropathy (Hansen *et al.*, 2004). The elevated serum MBL levels in type 1 diabetic patients with diabetic nephropathy were confirmed in another recent study, which also showed that elevated serum MBL-levels are seen even in microalbuminuric type 1 diabetic patients (Saraheimo *et al.*, 2004).

In a recent analysis of type II diabetic patients, high plasma levels of MBL were shown to predict albuminuria only when present in combination with high levels of CRP (Hansen *et al.*, 2006). Importantly, in type II diabetics, a high MBL level is a significant and independent risk factor for death, probably mainly explained by cardiovascular disease (Hansen *et al.*, 2006).

Whether differences in circulating MBL levels in diabetics are a contributing cause or a consequence of the development of microvascular complications cannot be established from cross-sectional studies, so Hovind *et al* (2005) conducted an 18-year follow up prospective study in type 1 diabetic patients and found MBL levels to be elevated even before the development of microalbuminuria, and the association of MBL to the development of microalbuminuria persisted after adjusting for known confounders. Such results suggest that MBL may play a causal role in the development of microvascular complications in diabetes. However, whether MBL is a risk factor and thereby actually involved in the pathogenesis of diabetic nephropathy or merely a risk marker associated with other factors of importance for disease progression is not yet established.

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Whether MBL is also found in the diabetic kidney is still not known, but it can be speculated that MBL ligands are present in diabetic kidneys, leading to a deposition of MBL in the kidney or even other target organs with deleterious effects. The mechanism could be that hyperglycaemia stimulates the production of N-acetylglucosamine through the hexosamine pathway, and as a consequence an abundance of various secretory and cell membrane glycoproteins are modified by N-linked glycosylation, enabling these proteins to be targets for MBL. (Saraheimo *et al.*, 2005)

In conclusion, these data show that MBL plays an unfavorable role in diabetic patients, most likely involving a pro-inflammatory role of MBL and complement activation at the vascular level.

1.6 MBL and diabetic retinopathy

It is now well established that the immune system is involved in the pathogenesis of diabetic retinopathy (Kaštelan *et al.*, 2007). Several studies have shown that activation of the complement system plays an important role in the pathogenesis of diabetic retinopathy. Gerl *et al.*, (2002) reported the evidence for extensive complement activation in the choriocapillaries of eyes with diabetic retinopathy. Using immunohistochemistry, these investigators found dense staining for membrane attack complex (MAC) and C3d, implicating complement activation in eyes with diabetic retinopathy. However, in the same study no positive staining was observed for C1q, C4 or MBL ,this observation did not allow the authors to conclude by which pathway (classical, lectin or alternative) the complement was activated. It is possible that the amounts of MBL present in these lesions were too low to be detected or that MBL escaped from the complexes after initiation of complement activation (Gerl *et al.*, 2002). Similar results were also reported

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by Zhang *et al.* (2002), who have shown that, although MAC staining co-localized with C3 in the retinal sections of the eyes obtained from diabetic patients; these sections did not stain for C1q and C4. Therefore the observed deposition of MAC may be the result of complement activation via the alternative pathway. Retinal samples from diabetic donors showed a significant reduction in CD55 and CD59, indicating the loss of regulatory mechanism may be the cause of increased complement activation (Zhang *et al.*, 2002). Similarly, increased complement activation and decrease in CD59 and CD55 levels were also observed in rats with streptozotocin-induced diabetes (Zhang *et al.*, 2002).

1.7 Possible mechanisms involving MBL, diabetes and its complications

The presence of hyperglycemia in diabetic patients, which is known to cause protein glycation, leads to the formation of advanced glycation end products (Brownlee *et al.*, 1988). Glycosylation changes may cause increased MBL autoreactivity (Malhotra *et al.*, 1995) and therefore explain why diabetic subjects are more susceptible to unfavorable effects of high MBL concentrations.

Moreover, emerging data indicate that increased glycation inactivates important membrane-bound complement regulatory proteins, such as CD59, that normally serve to prevent self-injury from insertion of the terminal membrane attack complex of complement (Qin *et al.*, 2004). Complement activation from any cause may thus have more widespread consequences in diabetic patients and contribute to the ongoing inflammation and microvascular and macrovascular complications of diabetes.

Based on *in vitro* findings, a proposed mechanism involving the binding of MBL to cell surface fructosamines and the activation of complement resulting from this high affinity

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binding was suggested by Fortpied *et al.* (2010). They demonstrate that complement is activated, as indicated by deposition of C3d, when fructoselysine plates are incubated with serum. The question is to know whether the binding of MBL to fructosamines occurs in diabetic patients. Although the cell surface fructosamine 'concentration' is difficult to evaluate, it is probably similar to the serum fructosamine concentration, i.e. about 1.6 mM in normal subjects and up to 2.4 mM in diabetic patients (Baker *et al.*, 1983). Complement deposits (C3d and C5b-9) have been found in the choriocapillaris of eyes of patients with diabetic retinopathy, but neither MBL nor C1q could be detected (Gerl *et al.*, 2002). Moreover, the observation that mouse deficient in MBL develops less diabetic nephropathy and cardiomyopathy, argues in favour of the contribution of the lectin pathway in the appearance of diabetic complications. In conclusion, the binding of MBL to fructosamines and the ensuing complement activation are a mechanism that deserves consideration among the potential mechanisms linking hyperglycaemia with diabetic complications. The cooperative character of this binding makes MBL/ fructosamine hypothesis attractive, as it may explain the high sensitivity of these complications to the mean blood glucose concentration.

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2. AIMS AND OBJECTIVES

1. To evaluate the level of MBL in the serum of diabetic patients compared to non-diabetics.

2. To explore possible relationship between MBL serum level and the presence of diabetic nephropathy

3. To explore possible relationship between MBL serum level and the presence of diabetic retinopathy.

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3. MATERIAL AND METHODS

3.1 MATERIALS

3.1.1 Reagents and pharmaceutical products

- Antibody coated Microwells with Frame purchased from Bioporto Diagnostics (Denmark)
- Sample Diluent purchased from Bioporto Diagnostics (Denmark)
- Seven MBL Calibrator solutions purchased from Bioporto Diagnostics (Denmark)
- 25x Wash Solution Concentrate purchased from Bioporto Diagnostics (Denmark)
- Biotinylated MBL Antibody purchased from Bioporto Diagnostics (Denmark)
- HRP-Streptavidin purchased from Bioporto Diagnostics (Denmark)
- TMB Substrate purchased from Bioporto Diagnostics (Denmark)
- Stop Solution purchased from Bioporto Diagnostics (Denmark)
- Sodium Hydroxide purchased form Biomed Diagnostics (Germany)
- Picric Acid purchased form Biomed Diagnostics (Germany)
- Creatinine standard 2mg/dl purchased from Biomed Diagnostics (Germany)

3.1.2 Instruments and apparatus

Biotek Microplate reader (USA), Centrifuge from Hettich (Germany), Photometer (Biosystems BTS 310 (Spain)), plate shaker Heidolph Unimax 2010 (Germany).

3.1.3 Patients and controls

An ethical committee approval was attained from The National Center for Diabetes, Endocrinology & Genetics. Outpatients who agreed to sign an informed consent form

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were enrolled and their serum samples were collected in sterile VACUETTE® Serum Clot Activator tubes (Greiner Bio-one (Austria)) and centrifuged for 7 min at speed of 4000 rpm. The supernatant was aspired into eppendorf tubes and stored by freezing (-20 °C) until analysis.

Sixty five patients diagnosed with type 2 diabetes were enrolled in the study; those were divided into two groups; with and without nephropathy. Nephropathy defined either as Crcl <60 ml/min or albumin in urine >20mg/l. The two groups were almost matched for age, gender, DM duration, presence of hypertension and HbA1C level.

Crcl was estimated after the measurement of serum creatinine using Cockroft-Gault equation for non obese patients and Salazar-Corcoran for obese patients.

Albuminuria measurement was done in the National Center for Diabetes, Endocrinology & Genetics using semiquantitative dipstick Micral® (results: negative, 20, 50, 100 mg/ml). Patients were considered to have microalbuminuria when elevated levels were seen in at least two of three collections done in 3-6 month period.

Fifteen healthy individuals with no diagnosed diabetes were enrolled in the study to function as control; those were matched for gender, age, BMI, family history of DM.

Inclusion criteria: patients diagnosed with type 2 diabetes. Diagnosis was made by a physician based on the ADA diagnosis criteria (table 1).

Exclusion criteria:

* Known secondary causes of diabetes such as polycystic ovary syndrome, pancreatitis, Cushing's disease, long term use of steroids.

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* Known causes of kidney damage such as congenital disorders, glomerulonephritis and drug induced kidney disease.

* Diseases that are known to be associated with high or low MBL levels such as rheumatoid arthritis, systemic lupus erythematosus and ongoing infections.

3.2 METHODS

3.2.1 Principle of the test

The assay is an ELISA performed in microwells coated with a monoclonal antibody against the MBL carbohydrate-binding domain. Bound MBL is detected with the same antibody that is labeled with biotin, followed by development with horseradish peroxidase (HRP)-conjugated streptavidin and incubation with a chromogenic substrate. Comparison of the assay results with molecular size chromatography of MBL immunoreactivity in individual human serum samples suggests that the monoclonal antibody used is selective for MBL oligomers when used as both capture and detection antibody. The assay is a four-step procedure:

Step 1. Aliquots of calibrators, diluted serum samples and any controls are incubated in microwells precoated with monoclonal antibody against MBL. MBL present in the solutions will bind to the antibody-coated wells via its carbohydrate-binding domains. Unbound material is removed by washing.

Step 2. Biotinylated monoclonal detection antibody is added to each test well and incubated. The detection antibody attaches to bound MBL oligomers via carbohydrate-binding domains that are not occupied by being bound down to the coat. Unbound detection antibody is removed by washing.

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Step 3. HRP-conjugated streptavidin is added to each test well and allowed to form a complex with the bound biotinylated antibody. Unbound conjugate is removed by washing.

Step 4. A chromogenic peroxidase substrate containing tetramethylbenzidine (TMB) is added to each test well. The bound HRP-streptavidin reacts with the substrate to generate a colored product. The enzymatic reaction is stopped chemically, and
the color intensity is read at 450 nm in an ELISA reader. The color intensity (optical density) is a function of the concentration of MBL oligomeric forms originally added to each well. The results for the calibrators are used to construct a calibration curve from which the concentrations of MBL in the test specimens are read.

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PRINCIPLE OF THE ASSAY PROCEDURE

MBL antibody



Plates are precoated with MBL antibody. The plates are ready for use

MBL



Diluted samples and calibrators are added to each well and incubated

Biotinylated MBL antibody



Biotinylated detection antibody is added to each well and incubated

Streptavidin – HRP



HRP-conjugated streptavidin is added to each well and incubated

TMB Substrate



Substrate is added to each well. Develop for 15 minutes in the dark

Stop solution

Stop Solution is added to each well. Read plate within 30 min.

Quantitative results are obtained by measuring the absorbances of the wells at 450 nm

Fig (3): Principle of the assay procedure (MBL Oligomer ELISA Kit, Bioporto Diagnostics)

3.2.2 Determination of MBL in serum samples

3.2.2.1 Reference curve solution preparation

All reagents were brought to room temperature before preparation. 100 µL of each of the different seven ready to use calibrator solutions were pipetted into corresponding positions in the microwells in duplicates and the assay procedure steps were performed.

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3.2.2.2 Wash solution preparation

Thirty milliliters of the wash solution provided with the kit were diluted to a final volume of 750 milliliters with distilled water in a graduated cylinder.

3.2.2.3 Serum samples preparation

Serum samples of both patients and controls were brought to room temperature. Five micro-liter (5 μL) of both patients' and controls' serum was added to 495 μL of sample diluent in Eppendorf tubes and vortexed (1:100 sample dilution).

3.2.2.4 Assay procedure steps

The following steps were followed for the measurement of MBL in serum samples:

1. One hundred micro-liters (100 μL) of each calibrator and diluted specimens of both patients and controls were pipetted into wells of the microplate provided with the kit. Duplicate well determination was performed for all of the above solutions. The wells were covered and incubated for 60 minutes at room temperature on a shaking platform.
2. The plate was inverted and emptied then the microwells were washed three times with 300 μL of diluted wash solution.
3. One hundred micro-liter (100 μL) of Biotinylated MBL Antibody (ready to use) were dispensed into each microwell using a multichannel pipette. The wells were covered and incubated for 60 minutes at room temperature on a shaking platform.
4. The plate was inverted and emptied then the microwells were washed three times with 300 μL of diluted wash solution.

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5. One hundred micro-liter (100 μL) of HRP-streptavidin Conjugate (ready to use) were dispensed into each microwell using a multichannel pipette. The wells were covered and incubated for 60 minutes at room temperature on a shaking platform.
6. The plate was inverted and emptied then the microwells were washed three times with 300 μL of diluted wash solution.
7. One hundred micro-liter (100 μL) of TMB Substrate (ready to use) were dispensed into each microwell using a multichannel pipette. The wells were covered and incubated for exactly 15 minutes at room temperature in the dark.
8. One hundred micro-liter (100 μL) of Stop Solution (ready to use) were dispensed into each microwell using a multichannel pipette maintaining the same pipetting sequence and rate as in step 7. The contents were mixed by gentle shaking for 20 seconds.
9. Absorbance of the wells was read at 450 nm in a microplate reader within 30 minutes (reference wavelength 650or 620 nm).

3.2.3 Determination of creatinine level in serum

Serum creatinine concentrations were determined by using the kinetic method. Each sample was analyzed to measure its creatinine concentration. A blank of both reagents (0.5 ml R_1 + 0.5 ml R_2) with distilled water (100 μL) was run to zero the spectrophotometer (R_1 was sodium hydroxide 0.4 mmol/L and R_2 was picric acid 38 mmol/L).

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One hundred micro-liters (100 μL) of creatinine standard (2mg/dl) was added to a pre mixed R_1 & R_2 reagents and then mixed well. The optical density was then measured after 0.5 and 2.5 minutes.

For each sample 0.5 ml of the first reagent R_1 (sodium hydroxide 0.4 mmol/L) were added to 0.5 ml of the second reagent, R_2 (picric acid 38 mmol/L) and mixed well.

One hundred micro-liters of the samples was added to the mixture of both reagents and vortexed. The optical density (OD) was then measured after 0.5 and 2.5 minutes beginning from the addition of the serum sample (100 μL) to the reagents using Photometer Biosystems BTS 310 (Spain).

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4. STATISTICAL ANALYSIS

All the data were analyzed using SPSS© software (version 17; SPSS, Inc, Chicago, IL). For all statistical analysis, a p-value of 0.05 or less was considered statistically significant. Continuous variables were presented as mean ± SD, and categorical variables were presented as frequencies and percentages. Unpaired sample t-test and nonparametric Mann–Whitney test were used for continuous variables. Chi-square or Fisher exact test were used to compare categorical variables.

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5. RESULTS

5.1 Data analysis

Seven ready to use reference solutions of MBL with the concentrations of 0.5, 1, 2, 5, 10, 20 and 40 ng/ml were used. The optical density (OD) of these solutions was measured in duplicates by Biotek Microplate reader. OD of the blank was then subtracted from those of the reference solutions. ODs of the reference solutions were then plotted against their corresponding concentrations. The relationship between concentration and the optical density was as shown in figure 4

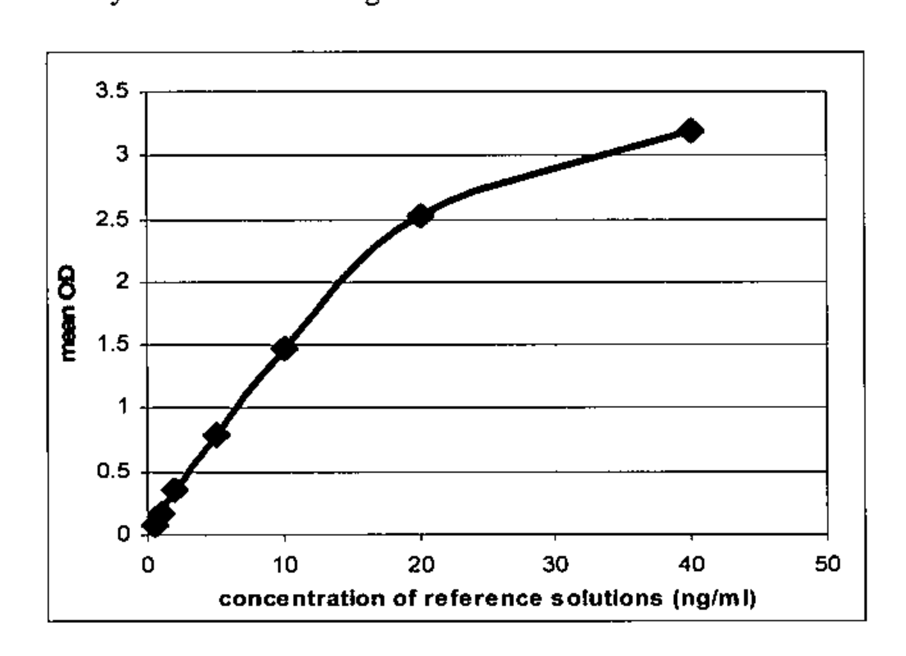


Fig (4): Calibration curve of the reference solution.

From the previous plot, the concentrations of MBL were generated (each sample was performed in duplicates, so the mean OD of each sample was calculated and the mean OD of the blank was subtracted from each value).

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Values were then multiplied by a dilution factor that was specified. Concentrations outside the range of the test (0.5-40 ng/ml) were specified as <0.5 or >40 ng/ml.

Absorbances of the creatinine standard (2 mg/dl) and specimens were measured using Biosystems BTS 310 photometer after 0.5 minutes (A_1) and after 2.5 minutes (A_2).

The concentration of creatinine in both patients' and controls' samples was determined according to the following equation $(A_2 - A_1)_{specimen} / (A_2 - A_1)_{standard} * 2$.

5.2 Patients' results

5.2.1 Demographic and clinical characteristics of patients and control group

Sixty five (65) out-patients from the National Center for Genetics, Diabetes and Endocrinology who were previously diagnosed with type 2 DM were enrolled in this study. Sixty three percent (63%) were females, 6 % were smokers, 18.5% were ex-smokers, 70.8% had positive family history for diabetes in first degree relatives, 73.8 % were hypertensive and 70.8 % had dyslipidemia. The mean age of patients was 55.9 ± 10.7 years. Fifteen (15) non-diabetic individuals were involved to function as a control group, these were matched for age, gender, BMI and family history for diabetes (mean age was 51.1 ± 8.5 years, 73 % females, 73% had positive family history of DM). Table (4) and table (5) summarize the demographic and clinical characteristics of both groups.

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Table (4) Demographic characteristics of patients (N=65)

Parameter	Diabetic patients, N (%)	Control group, N (%)	P-value
Number	65 (81)	15 (19)	
Age, years, mean (SD) [Age Range]	 55.88 (7.3) [39-75]	 51.1 (8.5) [42-70]	0.029
Gender Male Female	 24 (36.9) 41 (63.1)	 4 (26.7) 11 (73.3)	0.564
DM family history	46 (70.8)	11 (73.3)	1.000
Smoking	4 (0.06)	3 (20)	
BMI, mean (SD)	31.55 (4.15)	32 (5.97)	0.723

Table (5) Clinical characteristics of patients

Parameter	Diabetic patients, N (%)	Control group, N (%)	p-value
DM Duration, years (SD)	11.48 (5.36)		-
HbA1c, % mean (SD)	8.13 (1.66)	5.2 (0.32)	0.012
Retinopathy	27 (41.5)		-
Nephropathy	29 (44.6)		-
Hypertension	48 (73.8)	9 (60)	0.570
Dyslipidemia	46 (70.8)	10 (66.7)	0.755

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5.2.2 Drug therapy regimen of patients

The most commonly prescribed drugs were metformin, aspirin, statins, insulin, sulfonylureas, beta blockers, angiotensin converting enzyme inhibitors(ACEIs), angiotensin receptor blockers (ARBs) and calcium channel blockers (CCBs) as shown in Table (6).

Table (6) Drug therapy regimen of patients

Drug category	**Frequency N (%)**
Metformin	59 (90.8)
Aspirin	44 (67.7)
Statins	39 (60)
Insulin	37 (56.9)
Sulfonylureas	33 (50.8)
beta blockers	24 (36.9)
ARBs	22 (33.8)
ACEIs	19 (29.2)
CCBs	10 (15.4)

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5.2.3. MBL serum levels for patients and control

As shown in fig. (5), mean and median level of MBL in the diabetic group (mean: 1736 ±1405, median:1481) ng/ml were higher than those in the control group (mean:1190 ± 1291, median 526)ng/ml but the difference didn't reach statistical significance (*p*-value =0.167).

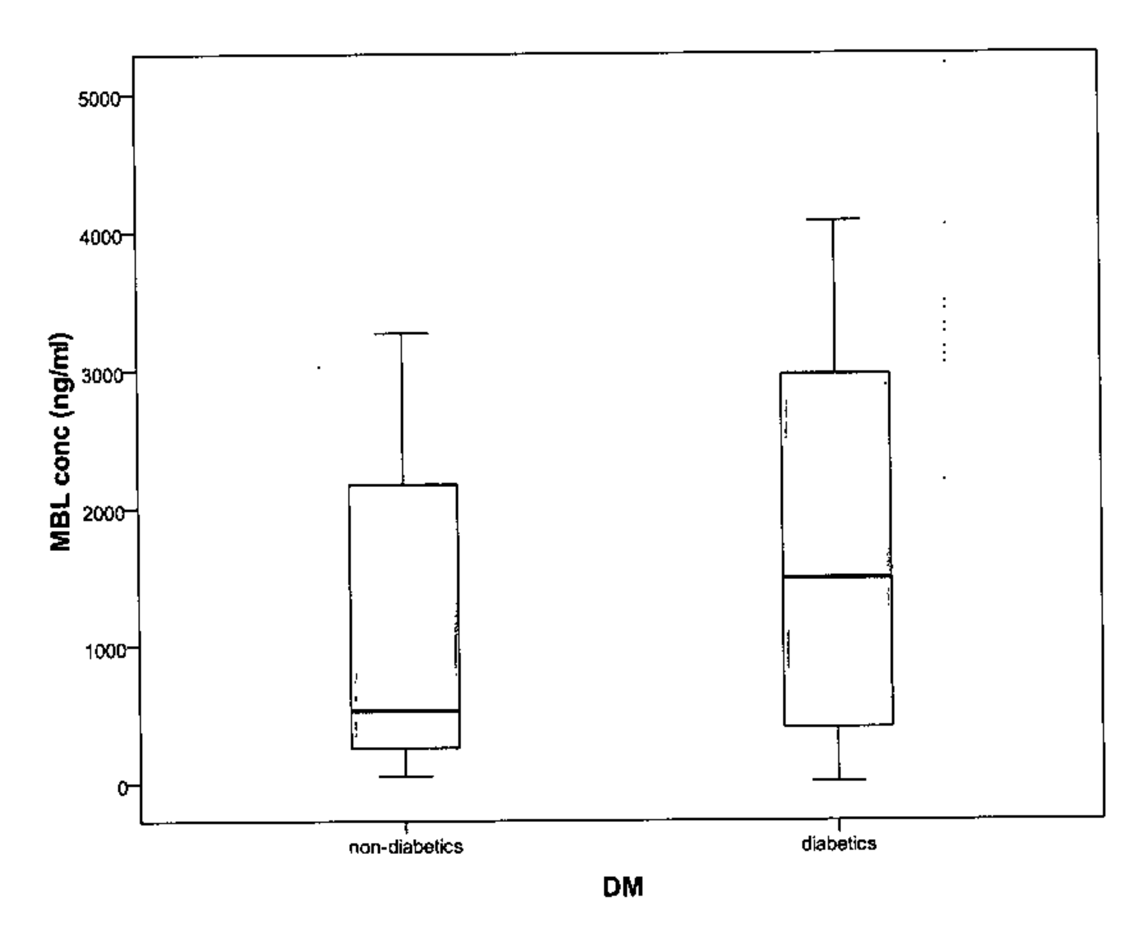


Fig (5) MBL serum levels in diabetic and non-diabetic patients

5.2.4 Association between MBL serum levels and gender

No significant difference in MBL level between males and females was found (fig (6)). (1894±1447 and 1493±1360 ng/ml respectively, *p*-value=0.223).

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Fig (6): MBL serum level in males and females

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5.2.5 Association between MBL serum levels and diabetic nephropathy

Diabetic patients were classified according to their kidney function to normal or nephropathic on the basis of their creatinine clearance (CrCl) and the presence of microalbuminurea (CrCl <60 ml/min or albuminurea> 20mg/L was considered to be nephropathic).

Basic demographic and clinical characteristics of both groups are summarized in table (7).

Mean and median MBL levels in nephropathic patients (mean: 2022±1399, median: 2276 ng/ml) was higher than that in non nephropathic (mean: 1504 ±1386, median: 1151 ng/ml) but the difference was not statistically significant (p= 0.141).

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However, when classified only according to the presence of albuminuria, MBL level in patients with microalbuminuria was significantly higher than that in normoalbuminuric patients (2187± 1405 vs. 1296± 1229 ng/ml, *p*-value = 0.010) as shown in figure (7). Basic demographic and clinical characteristics of both groups are summarized in table 8.

Table (7) Basic demographic and clinical characteristics of patients according to nephropathy

Variable	Patients with nephropathy (n=29)	Patients without nephropathy (n=36)	*p*-value
Age	58.5 ±4.9	53.8 ± 8.3	0.007
Gender (female)	17 (58.6%)	24 (66.7%)	0.504
BMI	32.1± 3.4	31.1± 4.7	0.359
Hypertension	22 (75.9%)	26 (72.2%)	0.740
HbA1c	8.2 ± 1.6	8.1 ± 1.8	0.841
Dyslipidemia	23 (79.3%)	23 (63.9%)	0.174
Taking ACE or ARB	20 (69%)	21(58.3%)	0.377
Duration of DM	12.1± 6.7	11±4.	0.452

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Table (8): Basic demographic and clinical characteristics of patients grouped according to microalbuminuria status

Variable	Patients with microalbuminuria (n=26)	Patients without microalbuminuria (n=37)	*p*-value
Age	57.5 ±4.9	54.8 ± 8.5	0.137
Gender (female)	13 (50.0%)	26 (70.3%)	0.103
BMI	31.5± 3.2	31.5± 4.7	0.986
Hypertension	18(69.2%)	28 (75.7%)	0.570
HbA1c	8.5 ± 1.6	7.8 ± 1.7	0.119
Dyslipidemia	20 (76.9%)	24 (64.9%)	0.305
Taking ACE or ARB	17 (65.4%)	23(62.2%)	0.794
Duration of DM	12.5± 6.8	10.8±4.0	0.211

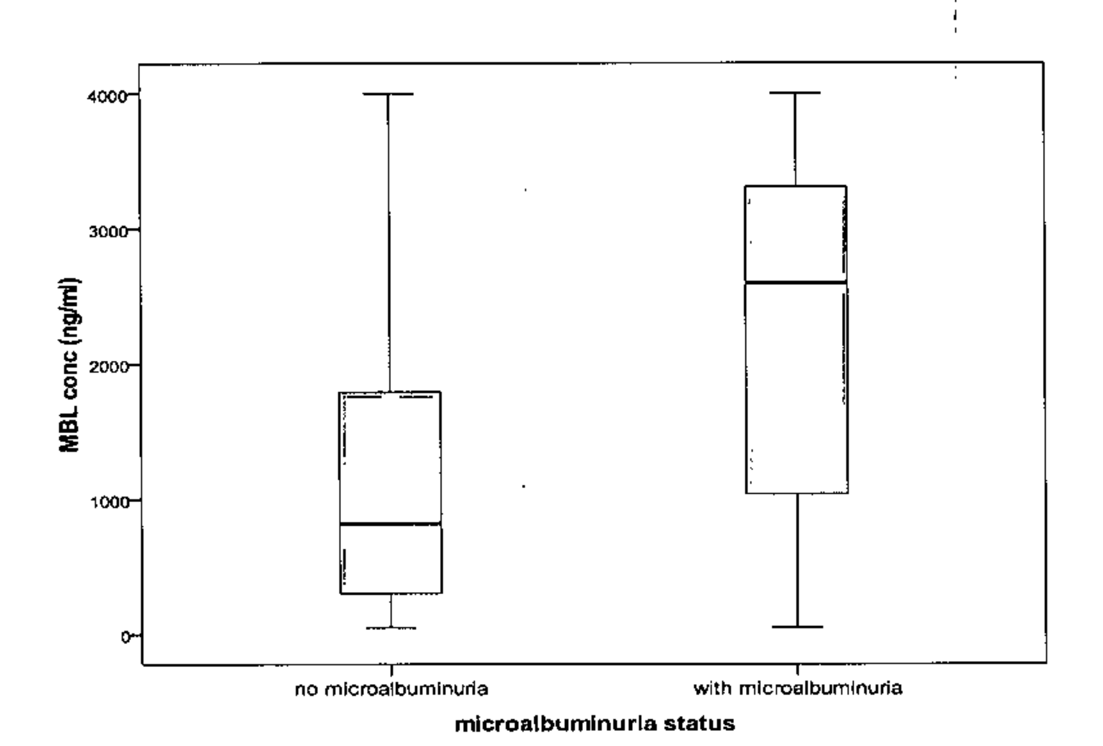


Fig. (7): MBL level in patients with and without microalbuminuria

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5.2.6 Association between MBL level and diabetic retinopathy

Diabetic patients were classified according to the presence of diabetic retinopathy (diagnosed by ophthalmologist). Basic demographic and clinical characteristics for both groups are summarized in table 9.

As shown in figure (8) mean MBL level in diabetic patients with retinopathy (2218± 1316) was higher than that in patients with no retinopathy (1392±1381) and the difference was statistically significant (p= 0.018). It is noteworthy to mention that age and duration of DM was significantly higher in the retinopathic group.

Table (9) Basic demographic and clinical characteristics of patients according to the presence of retinopathy

Variable	Patients with retinopathy (n=27)	Patients without retinopathy (n=38)	*p*-value
Age	58.7 ±6.7)	53.9±7.2	0.008
Gender (female)	13 (48%)	28 (73.7%)	0.036
BMI	31.6 ±4.2	31.5±4.2	0.920
Hypertension	20 (74.1%)	28 (73.7%)	0.501
HbA1c	8.4 ±1.9	8 ±1.4	0.351
Dyslipidemia	23 (85.2%)	23 (60.5%)	0.031
Duration of DM	14.7 ±5.7	9.2 ±3.8	<0.0005

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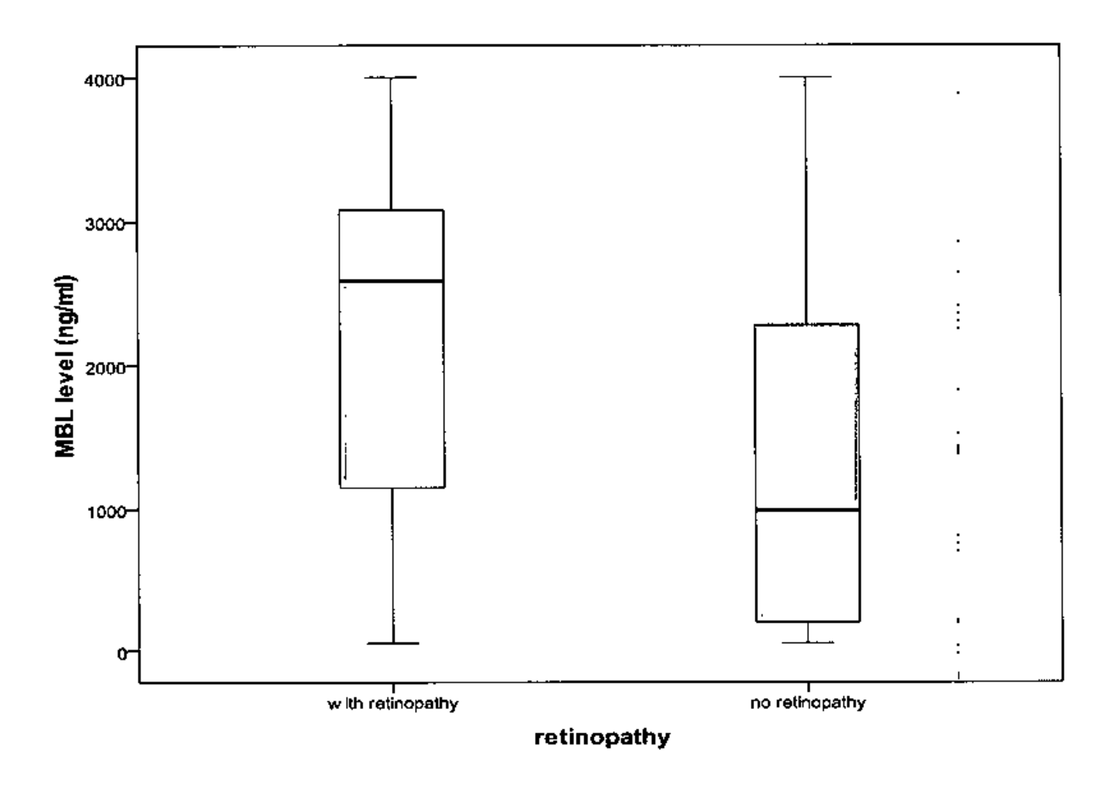


Fig (8): MBL serum levels in patients with and without retinopathy

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6. DISCUSSION

This study shows that MBL level in diabetic patients was higher than that in non-diabetics but the difference was not statistically significant. This is consistent with a study in Pima Indians and the Old Order Amish which showed significant association between functional variants of the MBL gene predicting high levels of MBL and increased risk of type 2 diabetes, the same study concluded that these variants are more likely to influence type 2 diabetes via an effect on insulin secretion rather than on insulin action, suggesting that inflammatory damage in pancreatic β-cell function may be involved (Muller *et al* 2010). Most of similar studies were concerned with type 1 DM rather than type 2, which has totally different pathogenesis.

The statistical significance was not evident in our study may be due to the small number of the non-diabetics group (n=15), so more studies with larger numbers are recommended to clarify association if present. In addition we should take into account that we studied the level of MBL protein rather than the genetic variability; the level might be affected by factors other than the specified genes studied. For example MBL was shown to be an acute phase reactant and can increase between 1.5 and three fold during the acute phase.

In this study no significant difference in MBL level was found between males and females. Cheng, SK *et al.* in a large study of Chinese adults and Ytting *et al* (2007) found similar results, but those were healthy adults rather than diabetics.

Association between MBL levels and genotypes and the development and progression of diabetic nephropathy was evaluated in many studies. Hansen *et al* (2010) and Kaunisto *et*

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al (2009) found that genotypes coding for high levels of MBL were found to be associated with higher incidence of micro and macro albuminuria.

Almost similar results was found in a recent analysis of type 2 diabetic patients, in which high plasma levels of MBL were shown to predict albuminuria when present in combination with high levels of CRP (Hansen *et al.*, 2006).

In our study significant association between MBL and microalbuminuria was found, but when taking nephropathy defined either as creatinine clearance < 60 ml/min or the presence of microalbuminuria MBL levels were higher in the nephropathic patients but this didn't reach statistical significance. Possible explanation is that the decrease in Crcl in some patients might be due to factors other than diabetes, and albuminuria is more related to nephropathy due to diabetes.

A limitation to our study was that the level of albumin in urine was measured using semiquantitative dipstick Micral® (results: negative, 20, 50, 100 mg/ml) rather than quantitative methods or 24-hour collected urine used in other studies.

Diabetic retinopathy is one of the leading causes of vision loss in middle-aged individuals (Moss *et al.*, 1998). It is now well established that the immune system is involved in the pathogenesis of diabetic retinopathy (Kaštelan *et al.*, 2007). Several studies have shown that activation of the complement system plays an important role in the pathogenesis of diabetic retinopathy. Complement deposits (C3d and C5b-9) have been found in the choriocapillaris of eyes of patients with diabetic retinopathy, but neither MBL nor C1q could be detected (Gerl *et al.*, 2002). This observation did not allow the authors to conclude by which pathway (classical, lectin or alternative) the complement was

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activated. It is possible that the amounts of MBL present in these lesions were too low to be detected or that MBL escaped from the complexes after initiation of complement activation.

In our study higher serum levels of MBL were associated with retinopathy (*p*-value = 0.018), such relationship has not been studied previously, but these results support the evidence of the role of complement system in retinopathy. Since age and duration of DM were higher in the group of patients suffering from retinopathy, this can be a possible confounder that limits our conclusion regarding this possible relationship so further studies should be in this area taking into account equal distribution of such confounders.

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7. CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

- High variability in serum MBL level among Jordanian population in general and diabetic patients in particular indicating underlying genetic variability in MBL2 gene.
- Our results show higher serum level of MBL in diabetic patients than matched non-diabetics suggesting possible contribution in type 2 DM pathogenesis, but this was not statistically significant.
- No difference in MBL level was found between males and females.
- Higher MBL levels were found in diabetic patients suffering from microalbuminuria suggesting possible risk factor for susceptibility to such complication.
- Higher MBL levels were found in diabetic patients suffering from retinopathy

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7.2 Recommendations for future work

- A larger cohort is recommended to evaluate MBL level in type 2 diabetic patients compared to non-diabetics with similar DM risk factors.
- Prospective studies are recommended to evaluate the effect of the level of MBL on various complications of diabetes such as nephropathy, retinopathy and cardiovascular risk taking in to account other confounders such as age and duration of DM.

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Appendix

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استمارة الإقرار بالعلم والموافقة

اسم الدراسة: - تقييم مستوى اللكتين الرابط لسكر المانوز و التركيب الجيني في مصل مرضى السكري

راعي الدراسة: الجامعة الأردنية.

الباحث الأول: د. ياسر البستنجي / الجامعة الأردنية / كلية الصيدلة / قسم الصيدلة الحيوية و السريرية

اسم الشخص المشارك :

(يمكنك السؤال عن أي نقطة غير واضحة في هذا النموذج)

طبيعة وغرض هذه الدراسة
نود منك المشاركة بشكل طوعي في دراسة بحثية ستستلزم سحب 10 ملم من دمك.
الغرض من هذه البحث هو دراسة مستوى اللكتين الرابط لسكر المانوز و التركيب الجيني و تأثير التباين على احتمالية الاصابة بالسكري.
المـــدة
ستقيد في دراسة لمدة يوم واحد فقط للحصول على عينة الدم.
عدد الأشخاص المشاركين في هذه الدراسة هو (100) شخص على الأقل من كلى المجموعتين (مرضى سكري وأصحاء).
تفسير الإجراءات المخبرية المتبعة:
إذا تطوعت سيكون ضرورياً أن يتم سحب حوالي 10 ملم من الدم .
سوف تحتاج لإعلام الطبيب عن تاريخك المرضي و عن الأدوية التي تتناولها.
لا يجب أن تشاركي إذا كنت تعلمين أو تشكين بأنك حامل.

الأخطار والمشقات المحتملة من المشاركة: لا يوجد

فوائد الدراسة:
.يتوقع في نهاية هذه الدراسة أن يكون هناك تحسن في توقع احتمالية حدوث السكري حسب التركيب الجيني و مستوى اللكتين و اختيار العلاج المناسب.

النفقات المالية:
سوف لن يتم دفع أي مبلغ من المال لك مقابل مشاركتك الطوعية في هذه الدراسة ولن يطلب منك المساهمة في أي من المصاريف المالية.

المشاركة الطوعية:
أنت تفهم أن المشاركة في هذه الدراسة طوعية. وأنت تفهم أن قرارك بعدم المشاركة في هذه الدراسة لن يؤثر على توفر الرعاية الطبية المستقبلية لك.

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<u>للاتصال أو الاستفسار:</u> إذا كان لديك أي استفسار أثناء الدراسة،يمكنك سؤال الباحث مباشرة

<u>سرية الدراسة؟</u>
المعلومات المستقاة من سجلاتك الطبية أثناء هذه الدراسة ستبقى سرية. قد يحتاج مجلس المراجعة الخاصة بالمؤسسة / اللجنة الأخلاقية أو أي من السلطات القانونية (مؤسسة الغذاء والدواء) للإطلاع على بعض هذه المعلومات.
بتوقيعك بالموافقة على هذه الاستمارة أنت توافق ضمنياً على الاطلاع على المعلومات في سجلك الطبي لغرض هذه الدراسة أو أي بحوث قادمة لها علاقة بالدراسة الحالية.

<u>حماية البيانات: كيف سيتم استخدام المعلومات المستقاة في هذه الدراسة؟</u>
البيانات الشخصل سيتم تحصيلها لأغراض بحثية فقط. البيانات في هذه الدراسة سوف يتم إرسالها حول العالم ولكن لن يتم الإشارة لك بالاسم ولن يتم التعريف عليك في أي تقرير أو منشور ولن يكون بالإمكان تعقب بياناتك إليك.
بمشاركتك في هذه الدراسة أنت توافق ضمنياً على عدم تقييد قدرة الباحثين على الوصول إلى بياناتك، وسيعمل الباحثون على اتخاذ كل ما هو مناسب وضروري لتأكيد أن معلوماتك الشخصية محمية.

<u>الموافقة على المشاركة في هذه الدراسة:</u>
أقر أنه:
* قد قرأت أو قُرئ لي بلغتي الأم المعلومات السابقة.
* محتوى ومعنى هذه المعلومات قد شُرح لي.
* كان لدي الفرصة للاستفسار عن هذه الدراسة واستمارة الإقرار هذه وقد تلقيت الإجابات التي أرضتني تماما.
* قد قرأت كل صفحات استمارة الإقرار بالعلم هذه وأقر أنني أفهم استمارة الإقرار والأخطار الموصوفة.

أنا بكامل حريتي واختياري:
* أوافق، وأعرض المشاركة في هذه الدراسة.
* بتوقيع استمارة الموافقة، أشهد أن كل المعلومات التي أعطيتها، متضمنة تاريخي الطبي، حقيقية وصحيحة على حد علمي.
* أفهم أنني سأتلقى نسخة عن استمارة الإقرار بالعلم.
* أصرح بتحرير سجلاتي الطبية إلى راعي الدراسة ولمؤسسة الغذاء والدواء.
* بتوقيعي على استمارة الإقرار هذه لم أتخلَ عن أي من حقوقي القانونية.

إمضاء المشارك (أو من هو مفوض عنه قانونياً) التاريخ

توقيع طالب الإقرار التاريخ

إمضاء الشاهد (إذا كان المشارك لا يستطيع القراءة) التاريخ

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المركز الوطني للسكري والغدد الصم والوراثة

NATIONAL CENTER FOR DIABETES ENDOCRINOLOGY & GENETICS

Ref. .... الرقم ج م/٩/٢٦٨

Date .... التاريخ ١٣/٢/٢٠١١م

الأستاذ الدكتور عميد كلية الصيدلة المحترم

تحية طيبة وبعد ،،،

ناقشت اللجنة المؤسسة للمركز الوطني للسكري والغدد الصم والوراثة توصيتكم بشأن إجراء الطالبة علا بشير سلام إجراء دراسة مسحية لمرضى السكري بهدف تقييم مستوى الكتين الرابط لسكر المانوز

Evaluation of mannose binding lectin level in serum of diabetic patients

وقد قررت اللجنة الموافقة على إجراء هذه الدراسة في جلستها التي عقدت بتاريخ 2011/2/1.

وتفضلوا بقبول فائق الاحترام

مقرر اللجنة

الأستاذ الدكتور محمد الخطيب

عمان - الأردن - شارع الملكة رانيا - هاتف: ٥٣٤٧٨١٠ / ٥٣٥٣٣٤٤ - فاكس: ٥٣٤٣٥٢٠ / ٥٣٥٣٣٧٦ - ص.ب : ١٣١٦٥ عمان ١١٩٤٢ الأردن

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تقييم مستوى اللكتين الرابط لسكر المانوز في مصل مرضى السكري

إعداد
علا بشير عبدالفتاح سلام

المشرف
الأستاذ الدكتور ياسر خليل البستنجي

المشرف المشارك
الدكتور محمد خليل محمد

706721

ملخص

يلعب اللكتين الرابط لسكر المانوز دورا اساسيا في المناعة الطبيعية, وقد وجدت بعض الدراسات انه يلعب دورا في الممانعة للانسولين واحتمالية الاصابة بالسكري ، كما ان بعض الدراسات وجدت علاقة بين مستوى هذا البروتين ومضاعفات مرض السكري.

تهدف هذه الدراسة الى تقييم مستوى اللكتين الرابط لسكر المانوز لدى مرضى السكري في الأردن، ومقارنته بالمستوى لدى الاشخاص غير المصابين بالسكري.

كما تهدف هذه الدراسة ايضا الى البحث فيما اذا كانت هنالك علاقة بين مستوى اللكتين الرابط لسكر المانوز وحدوث مضاعفات مرض السكري مثل امراض الكلى و امراض شبكية العين.

جمعت عينات الدم من خمسة وستين مريضا يعانون من السكري، وخمسة عشر لا يعانون من المرض، وتم قياس مستوى اللكتين الرابط لسكر المانوز في هذه العينات. كما تم قياس مستوى الكرياتينين لتقييم وظائف الكلى.

كشفت هذه الدراسة ان مستوى اللكتين الرابط لسكر المانوز لمرضى السكري اعلى منه لدى غير المصابين بمرض السكري، لكن الفرق لم يكن مهما من الناحية الاحصائية.

كما كشفت الدراسة عن ارتباط مستويات اللكتين الرابط لسكر المانوز العالية بمضاعفات السكري مثل امراض الكلى ممثلة بازدياد نسبة الالبيومين في البول ، وامراض شبكية العين باختلاف درجاتها.

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مما سبق يمكن التوصل الى ان المستويات العالية من اللكتين الرابط لسكر المانوز قد تلعب دورا مهما لدى مرضى السكري، وقد تساعد في التنبؤ باحتمالية حصول مضاعفات المرض، مما يتيح فرصة لاتخاذ تدابير وقائية أكثر صرامة.

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